kt. 0575/59131/JPW/WL

Application
for
United States Letters Patent

09/052750

To all whom it may concern:

Be it known that

I, Taka-Aki Sato
have invented certain new and useful improvements in

Gene Encoding NADE, $p75^{NTR}$-Associated Cell Death Executor and Uses Thereof

of which the following is a full, clear and exact description.

Dkt. 59131/JPW/WL

GENE ENCODING NADE, $p75^{NTR}$-ASSOCIATED CELL DEATH EXECUTOR AND USES THEREOF

This invention described herein was supported by National Institutes of Health grant R01-GM55147. Accordingly, the United States Government has certain rights in this invention.

Throughout this application various publications are referred to within parenthesis. Full citations for these publications may be found at the end of the specification immediately preceding the claims. The disclosures of these publications, in their entireties, are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Background of the Invention

The low-affinity neurotrophin receptor ($p75^{NTR}$) can mediate cell survival or cell death by NGF or another neurotrophins stimulation in neuronal cells (1, 2, 3). To elucidate $p75^{NTR}$-mediated signal transduction, the yeast two-hybrid system was employed to screen the mouse embryo cDNA libraries using the rat $p75^{NTR}$ICD (intracellular domain) as a target. One positive clone was identified and termed NADE ($p75^{NTR}$-associated cell death executor). NADE has a significant homology to human HGR74 protein (4) and does not have a typical biochemical motif except the consensus sequences of nuclear export signal (NES)(5) and ubiquitination (6). Expression of NADE mRNA was found highest in brain, heart, and lung. NADE specifically binds to $p75^{NTR}$ICD both *in vitro* and *in vivo*. Co-expression of NADE together with $p75^{NTR}$ dramatically induced Caspase-2 and Caspase-3 activities to cleave PARP (poly (ADP-ribose) polymerase) and fragmentation of nuclear DNA in 293T cells, but NADE without $p75^{NTR}$ did not show apoptosis, suggesting that NADE expression is necessary for $p75^{NTR}$-

mediated apoptosis but is not sufficient to trigger apoptosis. Moreover, NGF dependent recruitment of NADE to $p75^{NTR}$ICD was observed in a dose dependent manner and NADE significantly inhibits NF-kB activation. Interestingly, NADE protein is found to be ubiquitinated as a substrate for protein degradation pathway. Taken together, NADE is the first signal adaptor molecule identified in involvement of $p75^{NTR}$-mediated apoptosis, and it may play an important role in the pathogenesis of neurogenetic diseases.

Summary of the Invention

This invention provides an isolated nucleic molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor.

This invention provides a method of producing a polypeptide capable of binding a $p75^{NTR}$ receptor which comprises growing host cells selected from a group consisting of bacterial, plant, insect or mammalian cell, under suitable conditions permitting production of the polypeptide.

This invention provides an antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to an mRNA molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor.

This invention provides a purified polypeptide capable of binding a $p75^{NTR}$ receptor.

This invention provides a method of producing a polypeptide capable of binding a $p75^{NTR}$ receptor into a suitable vector which comprises: (a) inserting a nucleic acid molecule encoding the polypeptide capable of binding a $p75^{NTR}$ receptor into a suitable vector; (b) introducing the resulting vector into a suitable host cell; (c) selecting the introduced host cell for the expression of the polypeptide capable of binding a $p75^{NTR}$ receptor; (d) culturing the selected cell to produce the polypeptide capable of binding a $p75^{NTR}$ receptor; and (e) recovering the polypeptide capable of binding a $p75^{NTR}$ receptor produced.

This invention provides a method of identifying a compound capable of inhibiting binding between $p75^{NTR}$

receptor and a polypeptide capable of binding $p75^{NTR}$ receptor, where said binding forms a complex between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ receptor, comprising: a) contacting the compound under conditions permitting the binding of the polypeptide capable of binding $p75^{NTR}$ receptor and $p75^{NTR}$ receptor with the polypeptide capable of binding $p75^{NTR}$ receptor to form a mixture; b) contacting $p75^{NTR}$ receptor with the mixture from step a); and c) measuring the amount of complexed $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ receptor.

This invention provides a method of identifying a compound capable of inhibiting binding between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ receptor, where said binding forms a complex between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ receptor, comprising: a) contacting the compound under conditions permitting the binding of the polypeptide capable of binding $p75^{NTR}$ receptor and $p75^{NTR}$ receptor with the $p75^{NTR}$ receptor to form a mixture; b) contacting the polypeptide capable of binding a $p75^{NTR}$ receptor with the mixture from step a); and c) measuring the amount of complexed $p75^{NTR}$ receptor and a polypeptide.

This invention provides a method of inducing apoptosis in cells which comprises expressing a polypeptide capable of binding a $p75^{NTR}$ receptor in the cells.

This invention provides a method of inducing apoptosis in a subject which comprises expressing a polypeptide capable of binding a $p75^{NTR}$ receptor in the subject.

This invention provides a method of determining physiological effects of expressing varying levels of a polypeptide capable of binding a $p75^{NTR}$ receptor in

a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman mammals, each nonhuman mammal expressing a different amount of polypeptide capable of binding a $p75^{NTR}$ receptor.

This invention provides a method of inducing apoptosis of cells in a subject comprising administering to the subject the purified polypeptide capable of binding a $p75^{NTR}$ receptor in an amount effective to induce apoptosis.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of a polypeptide capable of binding a $p75^{NTR}$ receptor gene and $p75^{NTR}$ gene in the subject, an increase of the expression levels of the polypeptide capable of binding a $p75^{NTR}$ receptor gene and $p75^{NTR}$ gene indicating that the compound is an apoptosis inducing compound.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a cell with an appropriate amount of the compound; and (b) measuring the expression level of a polypeptide capable of binding a $p75^{NTR}$ receptor gene and $p75^{NTR}$ gene in the cell, an increase of the expression levels of the polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$ gene indicating that the compound is an apoptosis inducing compound.

This invention provides a method for screening cDNA libraries of a polypeptide capable of binding a $p75^{NTR}$ receptor sequence using a yeast two-hybrid system and using a $p75^{NTR}$ intracellular domain as a target.

This invention provides a method to induce caspase-2 and caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell by co-expression of a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$.

This invention provides a method to inhibit NF-κB activation in a cell with a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$.

This invention provides a method to detect a neurodegenerative disease in a subject by detecting expression levels of a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$.

This invention provides a transgenic nonhuman mammal which comprises an isolated nucleic acid, encoding a human HGR74 protein, which is a DNA molecule.

This invention provides a method of determining physiological effects of expressing varying levels of a human HGR74 protein in a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman mammal, each nonhuman mammal expressing a different amount of human HGR74 protein.

This invention provides a method of producing the isolated human HGR74 protein into a suitable vector which comprises: (a) inserting a nucleic acid molecule encoding a human HGR74 protein into a suitable vector; (b) introducing the resulting vector into a suitable host cell; (c) selecting the introduced host cell for the expression of the human HGR74 protein; (d) culturing the selected cell to produce the human HGR74 protein; and (e) recovering the human HGR74 protein produced.

This invention provides a method of inducing apoptosis of cells in a subject comprising administering to the subject the purified human HGR74 protein in an amount effective to induce apoptosis.

This invention provides a pharmaceutical composition comprising a purified polypeptide capable of binding a $p75^{NTR}$ receptor and a pharmaceutically acceptable carrier.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of human HGR74 protein gene and $p75^{NTR}$ gene in the subject, an increase of the expression levels of human HGR74 protein gene and $p75^{NTR}$ gene indicating that the compound is an apoptosis inducing compound.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a cell with an appropriate amount of the compound; and (b) measuring the expression level of human HGR74 gene and $p75^{NTR}$ gene in the cell, an increase of the expression levels of human HGR74 protein gene and $p75^{NTR}$ gene indicating that the compound is an apoptosis inducing compound.

This invention provides a method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a $p75^{NTR}$ intracellular domain as a target.

This invention provides a method to induce caspase-2 and caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell by co-expression of human HGR74 protein and $p75^{NTR}$.

This invention provides a method to inhibit NF-κB activation in a cell with human HGR74 protein and $p75^{NTR}$.

This invention provides a method to detect a neurodegenerative disease in a subject by detecting expression levels of a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$.

This invention provides a method of identifying a compound, which is an apoptosis inhibitor, said compound is capable of inhibiting specific binding between a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$ receptor, so as to prevent apoptosis which comprises: (a) contacting the polypeptide capable of binding a $p75^{NTR}$ receptor with a plurality of compounds under conditions permitting binding between a known compound previously shown to be able to displace the polypeptide capable of binding a $p75^{NTR}$ receptor and the $p75^{NTR}$ receptor and the bound $p75^{NTR}$ receptor to form a complex; and (b) detecting the displaced polypeptide capable of binding a $p75^{NTR}$ receptor or the complex formed in step (a), wherein the displacement indicates that the compound is capable of inhibiting specific binding between the polypeptide capable of binding a $p75^{NTR}$ receptor and the $p75^{NTR}$ receptor.

This invention provides a method of identifying a compound, which is an apoptosis inhibitor, said compound is capable of inhibiting specific binding between human HGR74 protein and $p75^{NTR}$ receptor, so as to prevent apoptosis which comprises: (a) contacting the human HGR74 protein with a plurality of compounds under conditions permitting binding between a known compound previously shown to be able to displace the human HGR74 protein and the $p75^{NTR}$ receptor and the

bound $p75^{NTR}$ receptor to form a complex; and (b) detecting the displaced human HGR74 protein or the complex formed in step (a), wherein the displacement indicates that the compound is capable of inhibiting specific binding between the human HGR74 protein and the $p75^{NTR}$ receptor.

Brief Description of Figures

The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

C=cytosine
A=adenosine
T=thymidine
G=guanosine

As used herein, amino acid residues are abbreviated as follows:

A=Alanine
C=Cysteine
D=Aspartic Acid
E=Glutamic Acid
F=Phenylalanine
G=Glycine
H=Histidine
I=Isoleucine
K=Lysine
L=Leucine
M=Methionine
N=Asparagine
P=Proline
Q=Glutamine
R=Arginine
S=Serine
T=Threonine
V=Valine
W=Tryptophan
Y=Tyrosine

Figure legends

Fig. 1 A-H Amino acid sequence and expression analysis

of NADE.

~~Figure~~ 1A

Amino acid alignment of mouse and human NADE ~~(HGR74)~~ (4) proteins. The dotted sequence is asparagine rich stretch. The asterisks indicate the leucine-rich nuclear export signal (NES)(5). The closed triangle indicates cysteine residue essential for dimmer formation. The prenylation sequence in C-termini is underlined.

~~Figure~~ 1B

Comparison of leucine-rich nuclear export signal ~~(NES)~~ (5) in various protein. The consensus sequence for NES are shadowed. Genbank accession numbers are: cZyxin, X69190; MAPKK, D13700; PKI-a, L02615; TFIIIA, M85211; RevHIV-1, AF075719; RanBP1, L25255; FMRP, L29074; Gle1, U68475; Human NADE, submitted; mouse NADE, submitted.

Figure 1C

Consensus sequence of ubiquitination signal.

Figure 1D

Northern blot analysis of NADE.

Figure 1E

Expression of endogenous NADE protein in SK-N-MC human neuroblastoma cells. SK-N-MC cell lysate treated with ALLN is immunoprecipitated by anti-NADE antibody, and subjected to immunoblotting by same antibody. Human NADE protein transiently expressed in 293T cells and untreated gels were used for controls. Heavy chain bands are resulted from antibodies using immunoprecipitation.

Figure 1F

Mutant analysis of mouse NADE protein A wild type NADE, muNADE(Cys102Ser), and muNADE(Cys121Ser) proteins transiently expressed in 293T cells were detected by immunoblotting with anti-NADE antibody. Transfection methods are described in material and methods. The cell lysate extracted from the 293T cells transfected with parental vector was used as a control.

Figure 1G-1 and 1G-2

Blast Search and comparison of mouse NADE nucleic acid sequence Figure 1G-1 (SEQ ID NO: __) and human protein HGR74 sequence

Figure 1H

Comparison of mouse NADE, human HGR74 protein and other homologous rat, mouse and human amino acid sequences

Fig. 2A-C NADE binds to $p75^{NTR}$ strongly *in vitro* and *in vivo*.

Figure 2A

In vitro binding assay of NADE and $p75^{NTR}$. *In vitro*-translated NADE protein was subjected to GST-pull down assay using a GST-$p75^{NTR}$ICD fusion protein. GST was used as a control.

Figure 2B

In vivo binding assay of NADE and $p75^{NTR}$. The cell lysates extracted from 293T cells co-transfected with Myc-tagged NADE and $p75^{NTR}$ were co-immunoprecipitated by anti-Myc antibody, and subjected to immunoblotting by anti-$p75^{NTR}$ antibody. The lysates from the cells transfected with each plasmid and a parental vector were used as controls. Transfection methods are described in material and methods.

Figure 2C

Interaction of NADE with $p75^{NTR}$ depending on NGF ligation. 293T cells co-transfected with Myc-tagged NADE and $p75^{NTR}$ were treated with NGF in various concentration as indicated. Upper panel; Immunoprecipitates of anti-Myc antibody (IgG1) from each sample were subjected to immunoblotting analysis by anti $p75^{NTR}$ antibody. Middle and lower panels indicated the expression level of $p75^{NTR}$ and NADE proteins by immunoblotting, respectively. The immunoprecipitate of anti-FLAG antibody (IgG1) was used as a control.

Fig. 3A-E Effect of NADE and $p75^{NTR}$ co-transfection on 293T cells.

Figure 3A

Morphological change caused by co-transfection of NADE and $p75^{NTR}$ in 293T cells transfected by each cDNA were observed 48 hours after transfection. The magnification was 200. Transfection methods are described in material and methods.

Figure 3B
TUNEL assay. Transfected 293T cells were stained by TUNEL method and analyzed by a flow cytometer. The percentages indicated are positive populations.

Figure 3C
DNA fragmentation analysis. DNAs from transfected 293T cells were checked by 1.5 % agarose gel electrophoresis.

Figure 3D
Inhibition of NF-κB activity by NADE. NF-κB activities in transfected cells were measured by E-selectin promoter-luciferase gene reporter assay. Luciferase activities were determined 24 hours after transfection and normalized on the basis of pRL-TK expression levels.

Figure 3E
Activation of Caspase-2 and 3 and degradation of PARP in co-transfected 293T cells. The cell extracts from 293T cells transfected by each cDNA as indicated were analyzed by immunoblotting with anti-Caspase-2, Caspase-3, and PAPP antibody. The level of a-tubulin was measured as a control.

Fig. 4A-D A conserved Rev-like NES in the C-terminus mediates nuclear export of NADE protein.

~~**Figure 4A**~~
At residues 88-100, the mouse ~~NADE NES~~ lies within the C-terminus. A mouse NADE is aligned with homologous sequences of NADE family members and the NES sequences of HIV Rev, MAPKK, cZyxin and PKI-a.

Figure 4B

Subcellular localization of a wild type mNADE-GFP and a control GFP vector was analyzed in transfected 293T cells.

Figure 4C

Effects of deletion mutants of NES motif on nuclear export of GFP-fused mouse NADE proteins. Both deletion mutants with or without NES indicate deletion-124 and delta 91-124, respectively.

Figure 4D

Effects of point mutations within the NES motif on nuclear export of GFP-fused mouse NADE proteins. The single or double amino acid substitutions were made at residue 94 and 97 (Leu to Ala). GFP-constructs were transiently transfected into 293T cells. The fixed cells were stained with TO-PRO-3 to visualize the nucleus and images of representative cell fields were captured on a confocal laser microscope. More than 1000 cells were analyzed for each construct.

Detailed Description of the Invention

The following standard abbreviations are used throughout the specification to indicate specific nucleotides:

C=cytosine
A=adenosine
T=thymidine
G=guanosine

As used herein, amino acid residues are abbreviated as follows:

A=Alanine
C=Cysteine
D=Aspartic Acid
E=Glutamic Acid
F=Phenylalanine
G=Glycine
H=Histidine
I=Isoleucine
K=Lysine
L=Leucine
M=Methionine
N=Asparagine
P=Proline
Q=Glutamine
R=Arginine
S=Serine
T=Threonine
V=Valine
W=Tryptophan
Y=Tyrosine

This invention provides an isolated nucleic molecule encoding a polypeptide capable of binding a $p75^{NTR}$

receptor. In an embodiment of the above described isolated nucleic molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor the isolated nucleic acid is a DNA molecule. In another embodiment of the above described isolated nucleic acid molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor the isolated nucleic acid is a cDNA molecule. In a further embodiment of the above described isolated DNA molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor the isolated nucleic acid is a RNA molecule. In an embodiment of the above described isolated nucleic acid molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor, the isolated nucleic acid is operatively linked to a promoter of RNA transcription. In yet another embodiment of the above described nucleic acid molecule, said isolated nucleic acid molecule encodes a neurotrophin associated cell death executor protein. In an embodiment of the above described nucleic acid molecule, said isolated nucleic acid molecule comprises a sequence of AATTG TCTAC GCATC CTTAT GGGGG AGCTG TCTAA C.

As used herein, "polypeptide" includes both peptides and proteins. "Peptide" means a polypeptide of fewer than 10 amino acid residues in length, and "protein" means a polypeptide of 10 or more amino acid residues in length. In this invention, the polypeptides may be naturally occurring or recombinant (i.e. produced via recombinant DNA technology), and may contain mutations (e.g. point, insertion and deletion mutations) as well as other covalent modifications (e.g. glycosylation and labeling [via biotin, streptavidin, fluoracine, and radioisotopes such as ^{131}I]). Moreover, each instant composition may contain more than a single

polypeptide, i.e., each may be a monomer (one polypeptide bound to a polymer) or a multimer (two or more polypeptides bound to a polymer or to each other).

The $p75^{NTR}$ receptor is a low affinity nerve growth factor (NGF) receptor with a low affinity to neurotrophins. $p75^{NTR}$ receptor has been implicated in the mediation of cell death and cell survival.

"Capable of binding" is defined as the ability of a protein or other peptide molecule capable of recognizing and interacting with a complementary receptor site, which can be another protein or other type of molecule.

The DNA molecules of the subject invention also include DNA molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The DNA molecules described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide capable of binding a $p75^{NTR}$ receptor, and as products for the large scale synthesis of the polypeptide capable of binding a $p75^{NTR}$ receptor, or fragments thereof, by a variety of recombinant techniques. The DNA molecule is useful for generating new cloning and expression vectors, transformed and transfected prokaryotic and eukaryotic host cells, and new and useful methods for cultured growth of such host cells capable of expression of the polypeptide capable of binding a $p75^{NTR}$ receptor or portions thereof and related products.

This invention provides a vector which comprises the isolated nucleic acid encoding a polypeptide capable of binding a $p75^{NTR}$ receptor, operatively linked to a promoter of RNA transcription. In an embodiment of the invention, where in the vector which comprises the isolated nucleic acid encoding a polypeptide capable of binding a $p75^{NTR}$ receptor, operatively linked to a promoter of RNA transcription is a plasmid. In another embodiment the above described isolated nucleic acid molecule which is a cDNA molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor, encodes a human or mouse protein. In yet another embodiment the above described isolated nucleic acid molecule is a cDNA molecule wherein the nucleic acid molecule encodes a polypeptide capable of binding a $p75^{NTR}$ receptor comprising the amino acid sequence set forth in Figure 1G-1 (SEQ ID NO: __). In a further embodiment the above described isolated nucleic acid molecule is a cDNA molecule wherein the

nucleic acid molecule encodes a polypeptide capable of binding a $p75^{NTR}$ receptor. In an embodiment of the above described isolated nucleic acid molecule which is a cDNA molecule wherein the nucleic acid molecule encodes a polypeptide capable of binding a $p75^{NTR}$ receptor which is a mouse, rat or human protein. In yet another embodiment of the above described isolated nucleic acid molecule which is a cDNA molecule, said isolated nucleic acid molecule comprises the nucleic acid sequence set forth in Figure 1G-1 (SEQ ID NO: __).

Numerous vectors for expressing the inventive proteins may be employed. Such vectors, including plasmid vectors, cosmid vectors, bacteriophage vectors and other viruses, are well known in the art. For example, one class of vectors utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MoMLV), Semliki Forest virus or SV40 virus. Additionally, cells which have stably integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow for the selection of transfected host cells. The markers may provide, for example, prototrophy to an auxotrophic host, biocide resistance or resistance to heavy metals such as copper. The selectable marker gene can be either directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. Additional elements may also be needed for

optimal synthesis of mRNA. These additional elements may include splice signals, as well as enhancers and termination signals. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG. Similarly, a eukaryotic expression vector includes a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well known in the art, for example the methods described above for constructing vectors in general.

These vectors may be introduced into a suitable host cell to form a host vector system for producing the inventive proteins. Methods of making host vector systems are well known to those skilled in the art.

Methods of introducing nucleic acid molecules into cells are well known to those of skill in the art. Such methods include, for example, the use of viral vectors and calcium phosphate co-precipitation.

This invention provides a host cell comprising the vector comprising the nucleic acid molecule of encoding a polypeptide capable of binding $p75^{NTR}$ receptor. In an embodiment the above described host cell is selected from a group consisting of a bacterial cell, a plant cell, and insect cell, and a mammalian cell.

The "suitable host cell" in which the nucleic acid molecule encoding is a polypeptide capable of binding

a $p75^{NTR}$ receptor capable of being expressed is any cell capable of taking up the nucleic acid molecule and stably expressing the polypeptide capable of binding a $p75^{NTR}$ receptor encoded thereby.

Suitable host cells include, but are not limited to, bacterial cells (including gram positive cells), yeast cells, fungal cells, insect cells and animal cells. Suitable animal cells include, but are not limited to HeLa cells, Cos cells, CV1 cells and various primary mammalian cells. Numerous mammalian cells may be used as hosts, including, but not limited to, the mouse fibroblast cell NIH-3T3 cells, CHO cells, HeLa cells, Ltk^- cells and COS cells. Mammalian cells may be transfected by methods well known in the art such as calcium phosphate precipitation, electroporation and microinjection.

This invention provides a method of producing a polypeptide having the biological activity of a polypeptide capable of binding a $p75^{NTR}$ receptor which comprises growing host cells selected from a group consisting of bacterial, plant, insect or mammalian cell, under suitable conditions permitting production of the polypeptide. In another embodiment of the above described method of producing a polypeptide having the biological activity of a polypeptide capable of binding a $p75^{NTR}$ receptor, the method further comprises the recovering of the produced polypeptide.

This invention provides an isolated nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the nucleic acid

molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor. In an embodiment of the above described isolated nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the nucleic acid molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor, said isolated nucleic acid molecule is a DNA molecule. In another embodiment of the above described isolated nucleic acid molecule of at least 15 contiguous nucleotides capable of specifically hybridizing with a unique sequence included within the sequence of the nucleic acid molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor, said isolated nucleic molecule is a RNA molecule.

This invention provides an isolated nucleic acid molecule capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule which is complementary to the nucleic acid molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor. In an embodiment the above described isolated nucleic acid molecule which is complementary to the nucleic acid molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor is a DNA molecule. In another embodiment the above described isolated nucleic acid molecule capable of specifically hybridizing with a nucleic acid molecule capable of specifically hybridizing with a unique sequence included within the sequence of a nucleic acid molecule which is complementary to the nucleic acid molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor is a RNA molecule.

One of ordinary skill in the art will easily obtain

unique sequences from the cDNA cloned in the polypeptide capable of binding a $p75^{NTR}$ receptor plasmid. Such unique sequences may be used as probes to screen various mammalian cDNA libraries and genomic DNAs, e.g. mouse, rat and bovine, to obtain homologous nucleic acid sequences and to screen different cDNA tissue libraries to obtain isoforms of the obtained nucleic acid sequences. Nucleic acid probes from the cDNA cloned in the polypeptide capable of binding a $p75^{NTR}$ receptor plasmid may further be used to screen other human tissue cDNA libraries to obtain isoforms of the nucleic acid sequences encoding polypeptide capable of binding a $p75^{NTR}$ receptor as well as to screen human genomic DNA to obtain the analogous nucleic acid sequences. The homologous nucleic acid sequences and isoforms may be used to produce the proteins encoded thereby.

As used herein, "capable of specifically hybridizing" means capable of binding to an mRNA molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor but not capable of binding to a polypeptide capable of binding a $p75^{NTR}$ receptor molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor.

This invention provides an antisense oligonucleotide having a nucleic acid sequence capable of specifically hybridizing to an mRNA molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor. In an embodiment of the above described antisense oligonucleotide, said antisense oligonucleotide has a nucleic acid sequence capable of specifically hybridizing to the isolated cDNA molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor. In another embodiment of the above described antisense oligonucleotide has a

nucleic acid sequence capable of specifically hybridizing to the isolated RNA molecule encoding a polypeptide capable of binding a $p75^{NTR}$ receptor.

This invention provides a purified a polypeptide capable of binding a $p75^{NTR}$ receptor. In an embodiment of the above described purified polypeptide capable of binding $p75^{NTR}$ receptor is encoded by the isolated nucleic acid encoding a polypeptide capable of binding a $p75^{NTR}$ receptor. In an embodiment the above described polypeptide capable of binding a $p75^{NTR}$ receptor is a fragment of the purified polypeptide capable of binding a $p75^{NTR}$ receptor. In another embodiment the above described purified polypeptide capable of binding a $p75^{NTR}$ receptor has substantially the same amino acid sequence as set forth in Figure 1G-1 (SEQ ID NO: __). In a further embodiment the above described purified polypeptide capable of binding a $p75^{NTR}$ receptor having an amino acid sequence as set forth in Figure 1G-1 (SEQ ID NO: __). In yet another embodiment the above described polypeptide capable of binding a $p75^{NTR}$ receptor has an amino acid sequence as set forth in Figure 1G-1 (SEQ ID NO: __). In a further embodiment, the above described polypeptide capable of binding a $p75^{NTR}$ receptor is a vertebrate polypeptide capable of binding a $p75^{NTR}$ receptor. In an embodiment of the above described polypeptide capable of binding a $p75^{NTR}$ receptor comprises a neurotrophin associated cell death executor protein. In yet another embodiment of the above described polypeptide capable of binding a $p75^{NTR}$ receptor comprises NCLRILMGELSN.

As used herein, purified polypeptides means the polypeptides free of any other polypeptides.

As used herein, a polypeptide capable of binding a $p75^{NTR}$ receptor having "substantially the same" amino acid sequences as set forth in Figure 1G-1 (SEQ ID NO: __) is encoded by a nucleic acid encoding a polypeptide capable of binding a $p75^{NTR}$ receptor, said nucleic acid having 100% identity in the homeodomain regions, that is those regions coding the protein, and said nucleic acid may vary in the nucleotides in the non-coding regions.

This invention provides a monoclonal antibody directed to an epitope of a polypeptide capable of binding a $p75^{NTR}$ receptor. In an embodiment the above described monoclonal antibody, said monoclonal antibody is directed to a mouse, rat or human polypeptide capable of binding a $p75^{NTR}$ receptor.

The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies, wholly synthetic antibodies, and fragments thereof. Optionally, an antibody can be labeled with a detectable marker. Detectable markers include, for example, radioactive or fluorescent markers.

This invention provides a polyclonal antibody directed to an epitope of the purified protein having the amino sequence as set forth in Figure 1G-1 (SEQ ID NO: __). In a further embodiment the above described monoclonal or polyclonal antibodies are directed to the polypeptide capable of binding a $p75^{NTR}$ receptor, having the amino sequence as set forth in Figure 1G-1

(SEQ ID NO: __).

Polyclonal antibodies may be produced by injecting a host animal such as rabbit, rat, goat, mouse or other animal with the immunogen of this invention, e.g. a purified mammalian polypeptide capable of binding a $p75^{NTR}$ receptor or a purified human polypeptide capable of binding a $p75^{NTR}$ receptor. The sera are extracted from the host animal and are screened to obtain polyclonal antibodies which are specific to the immunogen. Methods of screening for polyclonal antibodies are well known to those of ordinary skill in the art such as those disclosed in Harlow & Lane, Antibodies: A Laboratory Manual, (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY: 1988) the contents of which are hereby incorporated by reference.

The monoclonal antibodies may be produced by immunizing for example, mice with an immunogen. The mice are inoculated intra-peritoneally with an immunogenic amount of the above-described immunogen and then boosted with similar amounts of the immunogen. Spleens are collected from the immunized mice a few days after the final boost and a cell suspension is prepared from the spleens for use in the fusion.

In the practice of the subject invention any of the above-described antibodies may be labeled with a detectable marker. In one embodiment, the labeled antibody is a purified labeled antibody. As used in the subject invention, the term "antibody" includes, but is not limited to, both naturally occurring and non-naturally occurring antibodies. Specifically, the

term "antibody" includes polyclonal and monoclonal antibodies, and binding fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof.

Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. A "detectable moiety" which functions as detectable labels are well known to those of ordinary skill in the art and include, but are not limited to, a fluorescent label, a radioactive atom, a paramagnetic ion, biotin, a chemiluminescent label or a label which may be detected through a secondary enzymatic or binding step. The secondary enzymatic or binding step may comprise the use of digoxigenin, alkaline phosphatase, horseradish peroxidase, ß-galactosidase, fluorescein or steptavidin/biotin. Methods of labeling antibodies are well known in the art.

Determining whether the antibody forms such a complex may be accomplished according to methods well known to those skilled in the art. In the preferred embodiment, the determining is accomplished according to flow cytometry methods.

The antibody may be bound to an insoluble matrix such as that used in affinity chromatography. As used in the subject invention, isolating the cells which form a complex with the immobilized monoclonal antibody may be achieved by standard methods well known to those skilled in the art. For example, isolating may comprise affinity chromatography using immobilized antibody.

Alternatively, the antibody may be a free antibody. In this case, isolating may comprise cell sorting using free, labeled primary or secondary antibodies. Such cell sorting methods are standard and are well known to those skilled in the art.

The labeled antibody may be a polyclonal or monoclonal antibody. In one embodiment, the labeled antibody is a purified labeled antibody. The term "antibody" includes, by way of example, both naturally occurring and non-naturally occurring antibodies. Specifically, the term "antibody" includes polyclonal and monoclonal antibodies, and fragments thereof. Furthermore, the term "antibody" includes chimeric antibodies and wholly synthetic antibodies, and fragments thereof. The detectable marker may be, for example, radioactive or fluorescent. Methods of labeling antibodies are well known in the art.

This invention provides a method of inducing apoptosis in cells which comprises expressing polypeptide capable of binding a $p75^{NTR}$ receptor in the cells.

This invention provides a method of inducing apoptosis in a subject which comprises expressing a polypeptide capable of binding a $p75^{NTR}$ receptor in the subject. In a further embodiment of the method of inducing apoptosis in a subject where the subject is a rat, mouse or human.

As used herein, "subject" means any animal or artificially modified animal. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. In the preferred embodiment, the subject is a human.

This invention provides a transgenic nonhuman mammal which comprises an isolated nucleic acid, encoding a polypeptide capable of binding a $p75^{NTR}$ receptor, which is a DNA molecule. In an embodiment of the above described transgenic nonhuman mammal, the DNA encoding a polypeptide capable of binding a $p75^{NTR}$ receptor is operatively linked to tissue specific regulatory elements.

This invention provides a method of determining physiological effects of expressing varying levels of a polypeptide capable of binding a $p75^{NTR}$ receptor in a transgenic nonhuman mammal which comprises producing a panel of transgenic nonhuman mammals, each nonhuman mammal expressing a different amount of a polypeptide capable of binding a $p75^{NTR}$ receptor.

This invention provides a method of producing a polypeptide capable of binding a $p75^{NTR}$ receptor into a suitable vector which comprises: (a) inserting a nucleic acid molecule encoding the polypeptide capable of binding a $p75^{NTR}$ receptor into a suitable vector; (b) introducing the resulting vector into a suitable host cell; (c) selecting the introduced host cell for the expression of the polypeptide capable of binding a $p75^{NTR}$ receptor; (d) culturing the selected cell to produce the polypeptide capable of binding a $p75^{NTR}$ receptor; and (e) recovering the polypeptide capable of binding a $p75^{NTR}$ receptor produced.

This invention provides a method of inducing apoptosis of cells in a subject comprising administering to the subject the purified polypeptide capable of binding a $p75^{NTR}$ receptor in an amount effective to induce apoptosis. In an embodiment of the above described

method of inducing apoptosis of cells in a subject comprising administering to the subject the purified polypeptide capable of binding a $p75^{NTR}$ receptor in an amount effective to induce apoptosis, the subject is a mammal. In another embodiment of the above-described method of inducing apoptosis of cells in a subject, the subject is a mouse, rat or human.

As used herein "apoptosis" means programmed cell death of the cell. The mechanisms and effects of programmed cell death differs from cell lysis. Some observable effects of apoptosis are: DNA fragmentation and disintegration into small membrane-bound fragments called apoptotic bodies.

As used herein, "subject" means any animal or artificially modified animal. Artificially modified animals include, but are not limited to, SCID mice with human immune systems. In the preferred embodiment, the subject is a human.

This invention provides a pharmaceutical composition comprising a purified polypeptide capable of binding a $p75^{NTR}$ receptor and a pharmaceutically acceptable carrier.

The invention also provides a pharmaceutical composition comprising a effective amount of the polypeptides capable of binding a $p75^{NTR}$ receptor described above and a pharmaceutically acceptable carrier. In the subject invention an "effective amount" is any amount of above-described polypeptides capable of binding a $p75^{NTR}$ receptor which, when administered to a subject suffering from a disease or abnormality against which the proteins are determined to be potentially therapeutic, are effective, causes

reduction, remission, or regression of the disease or abnormality. In the practice of this invention the "pharmaceutically acceptable carrier" is any physiological carrier known to those of ordinary skill in the art useful in formulating pharmaceutical compositions.

In one preferred embodiment the pharmaceutical carrier may be a liquid and the pharmaceutical composition would be in the form of a solution. In another equally preferred embodiment, the pharmaceutically acceptable carrier is a solid and the composition is in the form of a powder or tablet. In a further embodiment, the pharmaceutical carrier is a gel and the composition is in the form of a suppository or cream. In a further embodiment the compound may be formulated as a part of a pharmaceutically acceptable transdermal patch.

A solid carrier can include one or more substances which may also act as flavoring agents, lubricants, solubilizers, suspending agents, fillers, glidants, compression aids, binders or tablet-disintegrating agents; it can also be an encapsulating material. In powders, the carrier is a finely divided solid which is in admixture with the finely divided active ingredient. In tablets, the active ingredient is mixed with a carrier having the necessary compression properties in suitable proportions and compacted in the shape and size desired. The powders and tablets preferably contain up to 99% of the active ingredient. Suitable solid carriers include, for example, calcium phosphate, magnesium stearate, talc, sugars, lactose, dextrin, starch, gelatin, cellulose, polyvinylpyrrolidine, low melting waxes and ion

exchange resins.

Liquid carriers are used in preparing solutions, suspensions, emulsions, syrups, elixirs and pressurized compositions. The active ingredient can be dissolved or suspended in a pharmaceutically acceptable liquid carrier such as water, an organic solvent, a mixture of both or pharmaceutically acceptable oils or fats. The liquid carrier can contain other suitable pharmaceutical additives such as solubilizers, emulsifiers, buffers, preservatives, sweeteners, flavoring agents, suspending agents, thickening agents, colors, viscosity regulators, stabilizers or osmo-regulators. Suitable examples of liquid carriers for oral and parenteral administration include water (partially containing additives as above, e.g. cellulose derivatives, preferably sodium carboxymethyl cellulose solution), alcohols (including monohydric alcohols and polyhydric alcohols, e.g. glycols) and their derivatives, and oils (e.g. fractionated coconut oil and arachis oil). For parenteral administration, the carrier can also be an oily ester such as ethyl oleate and isopropyl myristate. Sterile liquid carriers are useful in sterile liquid form compositions for parenteral administration. The liquid carrier for pressurized compositions can be halogenated hydrocarbon or other pharmaceutically acceptable propellent.

Liquid pharmaceutical compositions which are sterile solutions or suspensions can be utilized by for example, intramuscular, intrathecal, epidural, intraperitoneal or subcutaneous injection. Sterile solutions can also be administered intravenously. The compounds may be prepared as a sterile solid

composition which may be dissolved or suspended at the time of administration using sterile water, saline, or other appropriate sterile injectable medium. Carriers are intended to include necessary and inert binders, suspending agents, lubricants, flavorants, sweeteners, preservatives, dyes, and coatings.

The above described pharmaceutical composition comprising a polypeptide capable of binding a $p75^{NTR}$ receptor can be administered orally in the form of a sterile solution or suspension containing other solutes or suspending agents, for example, enough saline or glucose to make the solution isotonic, bile salts, acacia, gelatin, sorbitan monoleate, polysorbate 80 (oleate esters of sorbitol and its anhydrides copolymerized with ethylene oxide) and the like.

The above described pharmaceutical composition comprising a polypeptide capable of binding a $p75^{NTR}$ receptor can also be administered orally either in liquid or solid composition form. Compositions suitable for oral administration include solid forms, such as pills, capsules, granules, tablets, and powders, and liquid forms, such as solutions, syrups, elixirs, and suspensions. Forms useful for parenteral administration include sterile solutions, emulsions, and suspensions.

Optimal dosages to be administered may be determined by those skilled in the art, and will vary with the particular above described pharmaceutical composition comprising a polypeptide capable of binding a $p75^{NTR}$ receptor in use, the strength of the preparation, the mode of administration, and the advancement of the

disease condition or abnormality. Additional factors depending on the particular subject being treated will result in a need to adjust dosages, including subject age, weight, gender, diet, and time of administration.

As used herein, administering may be effected or performed using any of the various methods known to those skilled in the art. The administration may be intravenous, intraperitoneal, intrathecal, intralymphatical, intramuscular, intralesional, parenteral, epidural, subcutaneous; by infusion, liposome-mediated delivery, aerosol delivery; topical, oral, nasal, anal, ocular or otic delivery.

A method of identifying a compound capable of inhibiting binding between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ receptor comprising: a) contacting the compound with the polypeptide capable of binding to $p75^{NTR}$ receptor under conditions permitting the binding of the polypeptide capable of binding to $p75^{NTR}$ receptor and $p75^{NTR}$ receptor to form a complex; b) contacting the $p75^{NTR}$ receptor with the mixture from step a); and c) measuring the amount of the formed complexes or the unbound $p75^{NTR}$ receptor or the unbound polypeptide or any combination thereof. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a neurotrophin associated cell death executor. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a human HGR74 protein. In an embodiment of the above described method of

identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a musnade3a sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a hunade3a1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ a hunade3a2 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ a ratnad3a sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a ratnad3b sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a musnade3b sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a humnade1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding

$p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a ratnade1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a musnade1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a humnade2 sequence as defined on Figure 1H.

A method of identifying a compound capable of inhibiting binding between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ receptor, where said binding forms a complex between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ receptor, comprising: a) contacting the compound with the $p75^{NTR}$ receptor under conditions permitting the binding of the polypeptide capable of binding to $p75^{NTR}$ receptor and $p75^{NTR}$ receptor to form a complex; b) contacting the $p75^{NTR}$ receptor with the mixture from step a); and c) measuring the amount of the formed complexes or the unbound $p75^{NTR}$ receptor or the unbound polypeptide or any combination thereof.

In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a neurotrophin associated cell death executor protein. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a human HGR74 protein. In an embodiment of the above described method of

identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a musnade3a sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a hunade3a1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ a hunade3a2 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ a ratnad3a sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a ratnad3b sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a musnade3b sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a humnade1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding

$p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a ratnade1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a musnade1 sequence as defined on Figure 1H. In an embodiment of the above described method of identifying a compound capable of inhibiting between $p75^{NTR}$ receptor and a polypeptide capable of binding $p75^{NTR}$ where said polypeptide capable of binding $p75^{NTR}$ is a humnade2 sequence as defined on Figure 1H.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of polypeptide capable of binding a $p75^{NTR}$ receptor gene and $p75^{NTR}$ gene in the subject, an increase of the expression levels of a polypeptide capable of binding a $p75^{NTR}$ receptor gene and $p75^{NTR}$ gene indicating that the compound is an apoptosis inducing compound. In an embodiment of the above described method for identifying an apoptosis inducing compound comprising: a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of polypeptide capable of binding a $p75^{NTR}$ receptor gene and $p75^{NTR}$ gene in the subject, an increase of the expression levels of a polypeptide capable of binding a $p75^{NTR}$ receptor gene and $p75^{NTR}$ gene indicating that the compound is an apoptosis inducing compound, wherein the subject is a mammal. In an embodiment of the above-described method of identifying an apoptosis inducing compound, wherein the mammal subject is a mouse, rat or human.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a cell with an appropriate amount of the compound; and (b) measuring the expression level of polypeptide capable of binding a $p75^{NTR}$ receptor gene and $p75^{NTR}$ gene in the cell, an increase of the expression levels of polypeptide capable of binding a $p75^{NTR}$ receptor gene and $p75^{NTR}$ gene indicating that the compound is an apoptosis inducing compound.

An apoptosis inducing compound is defined as a compound which may be, but not limited to, antibodies, inorganic compounds, organic compounds, peptides, peptidomimetic compounds, polypeptides or proteins, fragments or derivatives which share some or all properities, e.g. fusion proteins, that induces apoptosis. The compounds may be naturally occurring and obtained by purification, or may be non-naturally occurring and obtained by synthesis.

This invention provides a method for screening cDNA libraries encoding a polypeptide capable of binding a $p75^{NTR}$ receptor sequence using a yeast two-hybrid system and using a $p75^{NTR}$ intracellular domain as a target. In an embodiment of the above described method for screening cDNA libraries for polypeptide capable of binding a $p75^{NTR}$ receptor sequence using a yeast two-hybrid system and using a $p75^{NTR}$ intracellular domain as a target, where the cDNA library is mammalian. In another embodiment of the above described method for screening cDNA libraries for a polypeptide capable of binding a $p75^{NTR}$ receptor using a yeast two-hybrid system and using a $p75^{NTR}$ intracellular domain as a target, where the cDNA library is mammalian and where the mammalian cDNA

library is derived from rat, mouse or human cDNA libraries. In an embodiment of the above described method for screening cDNA libraries for a polypeptide capable of binding a $p75^{NTR}$ receptor, using a yeast two-hybrid system and using a $p75^{NTR}$ intracellular domain as a target, where the $p75^{NTR}$ intracellular domain target is mammalian. In an embodiment of the above described method for screening cDNA libraries for a polypeptide capable of binding a $p75^{NTR}$ receptor using a yeast two-hybrid system and using a $p75^{NTR}$ intracellular domain as a target, where the $p75^{NTR}$ intracellular domain target is a rat, mouse or human $p75^{NTR}$ intracellular domain target.

This invention provides a method to induce caspase-2 and caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell by co-expression of a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$.

Caspases are members of the protease family, the mammalian homologs of the *Caenorhabiditis elegans* death gene ced-3, which are required for mammalian apoptosis. Increased levels of caspase-2 and caspase-3 have been linked to apoptosis. The caspases are cysteine aspartases that cleave their substrates at aspartate residues. To activate caspases, they need to be cleaved at aspartate residues and to form active heterodimers.

This invention provides a method to inhibit NF-κB activation in a cell with a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$.

NF-κB is a primary transcription factor which is

activated by external stimuli, and translocated to the nucleus where it binds to DNA and regulates gene transcription. In rat Schwann cells, the binding of nerve growth factor to $p75^{NTR}$ neurotrophin receptor, induces the activation of NF-κB in the absence of tyrosine kinase receptor A, and led to cell survival. NF-κB regulates the gene expression of various proteins including cell surface molecules and cytokines.

This invention provides a method to detect a neurodegenerative disease in a subject by detecting expression levels of a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$. In an embodiment of the above described method to detect a neurodegenerative disease in a subject by detecting expression levels of a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$, wherein the subject is a mammal. In another embodiment of the above described method to detect a neurodegenerative disease in a subject by detecting expression levels of a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$ wherein the mammal subject is mouse, rat or human.

This invention provides a transgenic nonhuman mammal which comprises an isolated nucleic acid, encoding a human HGR74 protein, which is a DNA molecule. In an embodiment of the above described transgenic nonhuman mammal, the DNA encoding a human HGR74 protein is operatively linked to tissue specific regulatory elements.

This invention provides a method of determining physiological effects of expressing varying levels of a human HGR74 protein in a transgenic nonhuman mammal

which comprises producing a panel of transgenic nonhuman mammal, each nonhuman mammal expressing a different amount of human HGR74 protein.

This invention provides a method of producing the isolated human HGR74 protein into a suitable vector which comprises: (a) inserting a nucleic acid molecule encoding a human HGR74 protein into a suitable vector; (b) introducing the resulting vector into a suitable host cell; (c) selecting the introduced host cell for the expression of the human HGR74 protein; (d) culturing the selected cell to produce the human HGR74 protein; and (e) recovering the human HGR74 protein produced.

This invention provides a method of inducing apoptosis of cells in a subject comprising administering to the subject the purified human HGR74 protein in an amount effective to induce apoptosis. In an embodiment of the above described method of inducing apoptosis of cells in a subject comprising administering to the subject the purified human HGR74 in an amount effective to induce apoptosis, the subject is a mammal. In another embodiment of the above-described method of inducing apoptosis of cells in a subject, the subject is a mouse, rat or human.

This invention provides a pharmaceutical composition comprising a purified human HGR74 protein and a pharmaceutically acceptable carrier.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of

human HGR74 protein gene and $p75^{NTR}$ gene in the subject, an increase of the expression levels of human HGR74 protein gene and $p75^{NTR}$ gene indicating that the compound is an apoptosis inducing compound. In an embodiment of the above described method for identifying an apoptosis inducing compound comprising: a) contacting a subject with an appropriate amount of the compound; and (b) measuring the expression level of human HGR74 protein gene and $p75^{NTR}$ gene in the subject, an increase of the expression levels of human HGR74 protein gene and $p75^{NTR}$ gene indicating that the compound is an apoptosis inducing compound, wherein the subject is a mammal. In an embodiment of the above-described method of identifying an apoptosis inducing compound, wherein the mammal subject is a mouse, rat or human.

This invention provides a method for identifying an apoptosis inducing compound comprising: (a) contacting a cell with an appropriate amount of the compound; and (b) measuring the expression level of human HGR74 gene and $p75^{NTR}$ gene in the cell, an increase of the expression levels of human HGR74 protein gene and $p75^{NTR}$ gene indicating that the compound is an apoptosis inducing compound.

This invention provides a method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a $p75^{NTR}$ intracellular domain as a target. In an embodiment of the above described method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a $p75^{NTR}$ intracellular domain as a target, where the cDNA library is mammalian. In an embodiment of the above described method for screening cDNA libraries human

HGR74 sequence using a yeast two-hybrid system using a $p75^{NTR}$ intracellular domain as a target, where the cDNA library is mammalian and where the mammalian cDNA library is derived from rat, mouse or human cDNA libraries. In another embodiment of the above described method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a $p75^{NTR}$ intracellular domain as a target, where the $p75^{NTR}$ intracellular domain target is mammalian. In an embodiment of the above described method for screening cDNA libraries human HGR74 sequence using a yeast two-hybrid system using a $p75^{NTR}$ intracellular domain as a target, where the $p75^{NTR}$ intracellular domain target is a rat, mouse or human $p75^{NTR}$ intracellular domain target.

This invention provides a method to induce caspase-2 and caspase-3 activity to cleave poly (ADP-ribose) polymerase and fragment nuclear DNA in a cell by co-expression of human HGR74 protein and $p75^{NTR}$.

This invention provides a method to inhibit NF-κB activation in a cell with human HGR74 protein and $p75^{NTR}$.

This invention provides a method to detect a neurodegenerative disease in a subject by detecting expression levels of polypeptide capable of binding a $p75^{NTR}$ receptor a and $p75^{NTR}$. In an embodiment of the above described method to detect a neurodegenerative disease in a subject by detecting expression levels of polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$, wherein the subject is a mammal. In another embodiment of the above described method to detect a neurodegenerative disease in a subject by detecting

expression levels of polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$, wherein the subject is a mammal wherein the mammal is human.

This invention provides a method of identifying a compound, which is an apoptosis inhibitor, said compound is capable of inhibiting specific binding between a polypeptide capable of binding a $p75^{NTR}$ receptor and $p75^{NTR}$ receptor, so as to prevent apoptosis which comprises: (a) contacting the polypeptide capable of binding a $p75^{NTR}$ receptor with a plurality of compounds under conditions permitting binding between a known compound previously shown to be able to displace the polypeptide capable of binding a $p75^{NTR}$ receptor and the $p75^{NTR}$ receptor and the bound $p75^{NTR}$ receptor to form a complex; and (b) detecting the displaced polypeptide capable of binding a $p75^{NTR}$ receptor or the complex formed in step (a), wherein the displacement indicates that the compound is capable of inhibiting specific binding between the polypeptide capable of binding a $p75^{NTR}$ receptor and the $p75^{NTR}$ receptor. In another embodiment of the above described method, wherein the inhibition of specific binding between the polypeptide capable of binding a $p75^{NTR}$ receptor and the $p75^{NTR}$ receptor affects the transcription activity of a reporter gene. In a further embodiment of the above described method, wherein step (b) the displaced polypeptide capable of binding a $p75^{NTR}$ receptor or the complex is detected by comparing the transcription activity of a reporter gene before and after the contacting with the compound in step (a), where a change of the activity indicates that the specific binding between the polypeptide capable of binding a $p75^{NTR}$ receptor and the $p75^{NTR}$ receptor is inhibited and the polypeptide capable of

binding a $p75^{NTR}$ receptor is displaced. In an embodiment of the above described method, wherein the $p75^{NTR}$ receptor is bound to a solid support. In a further embodiment of the above described method, wherein the compound is bound to a solid support. In an embodiment of the above described method, wherein the compound comprises an antibody, an inorganic compound, an organic compound, a peptide, a peptidomimetic compound, a polypeptide or a protein. In an embodiment of the above described method, wherein the contacting of step (a) is in vitro. In a further embodiment of the above method, wherein the contacting of step (a) is in vivo. In an embodiment of the above method, wherein the contacting of step (a) is in a yeast cell. In an embodiment of the above method, wherein the contacting or step (a) is in a mammalian cell. In an embodiment of the above method, wherein the polypeptide capable of binding a $p75^{NTR}$ receptor is a cell surface receptor. In an embodiment of the above method, wherein the cell-surface receptor is the p75 receptor.

As used herein, the "transcription activity of a reporter gene" means that the expression level of the reporter gene will be altered from the level observed when the signal-transducing protein and the cytoplasmic protein are bound. One can also identify the compound by detecting other biological functions dependent on the binding between the signal-transducing protein and the cytoplasmic protein. Examples of reporter genes are numerous and well-known in the art, including, but not limited to, histidine resistant genes, ampicillin resistant genes, β-galactosidase gene.

Further the cytoplasmic protein may be bound to a solid support. Also the compound may be bound to a solid support and comprises an antibody, an inorganic compound, an organic compound, a peptide, a peptidomimetic compound, a polypeptide or a protein.

An example of the method is provided <u>infra</u>. One can identify a compound capable of inhibiting specific binding between the signal-transducing protein and the cytoplasmic protein using direct methods of detection such as immuno-precipitation of the cytoplasmic protein and the compound bound to a detectable marker. Further, one could use indirect methods of detection that would detect the increase or decrease in levels of gene expression. As discussed <u>infra</u>, one could construct synthetic peptides fused to a LexA DNA binding domain. These constructs would be transformed into the L40-strain with an appropriate cell line having an appropriate reporter gene. One could then detect whether inhibition had occurred by detecting the levels of expression of the reporter gene. In order to detect the expression levels of the reporter gene, one skilled in the art could employ a variety of well-known methods, e.g. two-hybrid systems in yeast, mammals or other cells.

Further, the contacting of step (a) may be <u>in vitro</u>, <u>in vivo</u>, and specifically in an appropriate cell, e.g. yeast cell or mammalian cell. Examples of mammalian cells include, but not limited to, the mouse fibroblast cell NIH 3T3, CHO cells, HeLa cells, Ltk^- cells, Cos cells, etc.

Other suitable cells include, but are not limited to, prokaryotic or eukaryotic cells, e.g. bacterial cells

(including gram positive cells), fungal cells, insect cells, and other animals cells.

This invention provides a method of identifying a compound, which is an apoptosis inhibitor, said compound is capable of inhibiting specific binding between human HGR74 protein and $p75^{NTR}$ receptor, so as to prevent apoptosis which comprises: (a) contacting the human HGR74 protein with a plurality of compounds under conditions permitting binding between a known compound previously shown to be able to displace the human HGR74 protein and the $p75^{NTR}$ receptor and the bound $p75^{NTR}$ receptor to form a complex; and (b) detecting the displaced human HGR74 protein or the complex formed in step (a), wherein the displacement indicates that the compound is capable of inhibiting specific binding between the human HGR74 protein and the $p75^{NTR}$ receptor. In an embodiment of the above described method, wherein the inhibition of specific binding between the human HGR74 protein and the $p75^{NTR}$ receptor affects the transcription activity of a reporter gene. In a further embodiment of the above described method, wherein step (b) the displaced human HGR74 protein or the complex is detected by comparing the transcription activity of a reporter gene before and after the contacting with the compound in step (a), where a change of the activity indicates that the specific binding between the human HGR74 protein and the $p75^{NTR}$ receptor is inhibited and the human HGR74 protein is displaced. In an embodiment of the above described method, wherein the $p75^{NTR}$ receptor is bound to a solid support. In a further embodiment of the above described method, wherein the compound is bound to a solid support. In an embodiment of the above described method, wherein the compound comprises an

antibody, an inorganic compound, an organic compound, a peptide, a peptidomimetic compound, a polypeptide or a protein. In an embodiment of the above described method, wherein the contacting of step (a) is in vitro. In a further embodiment of the above method, wherein the contacting of step (a) is in vivo. In an embodiment of the above method, wherein the contacting of step (a) is in a yeast cell. In an embodiment of the above method, wherein the contacting or step (a) is in a mammalian cell. In an embodiment of the above method, wherein the human HGR74 protein is a cell surface receptor. In an embodiment of the above method, wherein the cell-surface receptor is the p75 receptor.

As used herein, the "transcription activity of a reporter gene" means that the expression level of the reporter gene will be altered from the level observed when the signal-transducing protein and the cytoplasmic protein are bound. One can also identify the compound by detecting other biological functions dependent on the binding between the signal-transducing protein and the cytoplasmic protein. Examples of reporter genes are numerous and well-known in the art, including, but not limited to, histidine resistant genes, ampicillin resistant genes, β-galactosidase gene.

Further the cytoplasmic protein may be bound to a solid support. Also the compound may be bound to a solid support and comprises an antibody, an inorganic compound, an organic compound, a peptide, a peptidomimetic compound, a polypeptide or a protein.

An example of the method is provided infra. One can

identify a compound capable of inhibiting specific binding between the signal-transducing protein and the cytoplasmic protein using direct methods of detection such as immuno-precipitation of the cytoplasmic protein and the compound bound to a detectable marker. Further, one could use indirect methods of detection that would detect the increase or decrease in levels of gene expression. As discussed infra, one could construct synthetic peptides fused to a LexA DNA binding domain. These constructs would be transformed into the L40-strain with an appropriate cell line having an appropriate reporter gene. One could then detect whether inhibition had occurred by detecting the levels of expression of the reporter gene. In order to detect the expression levels of the reporter gene, one skilled in the art could employ a variety of well-known methods, e.g. two-hybrid systems in yeast, mammals or other cells.

Further, the contacting of step (a) may be in vitro, in vivo, and specifically in an appropriate cell, e.g. yeast cell or mammalian cell. Examples of mammalian cells include, but not limited to, the mouse fibroblast cell NIH 3T3, CHO cells, HeLa cells, Ltk$^-$ cells, Cos cells, etc.

Other suitable cells include, but are not limited to, prokaryotic or eukaryotic cells, e.g. bacterial cells (including gram positive cells), fungal cells, insect cells, and other animals cells.

In order to facilitate an understanding of the material which follows, certain frequently occurring methods and/or terms are best described in Sambrook, et al., 1989.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Results and Discussions

The $p75^{NTR}$ is the first-isolated neurotrophin receptor and the member of TNFR (tumor necrosis factor receptor) family (7, 8). However, its functional role and signaling pathway has remained largely unclear (9). The existence of $p75^{NTR}$ICD binding proteins have been implicated since $p75^{NTR}$ICD does not have a typical biochemical motif except a C-terminal region well conserved to a type 2 death domain (10). Recently, it has been reported that TRAF6 is involved in $p75^{NTR}$-mediated signal transduction(11). To further identify the $p75^{NTR}$ICD binding proteins, we screened the mouse cDNA libraries by yeast two-hybrid system using a rat $p75^{NTR}$ICD as a target and one of positive clones was identified as a $p75^{NTR}$-associated cell death executor, NADE.

NADE consists of 124 amino acids and its molecular weight is calculated to 14,532 dalton. NADE is a hydrophilic and acidic protein, and the estimated p*I* value is 5.97. A BLAST search revealed that NADE has significant homology to a known human protein HGR74(4) (Fig. 1a), and does not have a significant motif except the leucine rich nuclear export signal (NES) (5) (Fig. 1b) and ubiquitination sequences (6) (Fig. 1c) HGR74 was previously reported as an abundant mRNA expressed in human ovarian granulosa cells, however, its functional role is still unknown. The homology of these two proteins except the asparagine rich stretch (a. a. 36-48) of NADE is 92.8%, therefore we conclude that HGR74 is a human homolog of mouse NADE.

Northern blot analysis is revealed that NADE mRNA (1.3 kbp) is found highest in several tissues including brain, heart, and lung (Fig. 1d). We could also

detect a low level of mRNA expression in stomach, small intestine, and muscle by a long exposure (data not shown). But there was no expression in liver. The additional large band (3.0 kbp) was also observed in testis, suggesting the existence of the alternative splicing form. The endogenous NADE protein was also confirmed in human neuroblastoma cell line, SK-N-MC by immunoprecipitation using the anti-NADE antibody (Fig. 1e). Interestingly, in SK-N-MC, PC12 and PCNA cells, NADE protein can be detected only in the presence of the ubiquitin inhibitor such as ALLN, suggesting that NADE is modified by ubiquitin conjugating system leading to subsequent degradation by the proteasome. The molecular size of NADE is estimated to 22 kDa by the SDS-PAGE, and this size seems to be slightly larger than the molecular weight predicted from nucleotide sequence. But the gap of molecular size might be caused by its low p*I* value or post-translational modification in a potential prenylation site (Fig. 1a). The overexpressed NADE protein in 293T cells showed the two bands, 22 kDa ad 44 kDa in SDS-PAGE under the reduced condition at 100 mM dithiothreitol (Fig. 1f). To clarify this question, two NADE mutants were constructed and expressed in 293T cells. Since NADE has two cysteine residues at sequence positions 102 and 121, we replaced the each cysteine with the serine residue. Western blot analysis revealed that the molecular weight of muNADE (Cys121Ser) is identical to a wild type, on the other hand, muNADE (Cys102Ser) showed the only smaller band of 22 kDa (Fig. 1g). These results strongly suggested that NADE can heterodimerize by the disulfide bound at the Cys102, and resulted in the 44 kDa band.

In vitro-translated mouse NADE protein and *E. coli*-expressed GST- $p75^{NTR}$ICD fusion protein were used for *in vitro* GST pull down assay. In this assay, the NADE

protein showed the strong binding activity to GST-P $p75^{NTR}$ICD (Fig. 2a). To investigate the *in vivo* binding activity, the Myc-tagged NADE and $p75^{NTR}$ were co-expressed in 293T cells and subjected to the co-immunoprecipitation experiment. The results clearly showed that NADE could bind to a full length of $p75^{NTR}$ *in vivo* very strongly (Fig. 2b) and the recruitment of NADE protein to $p75^{NTR}$ICD was detected in a dose dependent of NGF (Fig. 2c), suggesting that NADE protein is a putative signal transducing protein interacting with $p75^{NTR}$ICD. Furthermore, our mapping studies revealed that NADE protein interacts with the cell death domain (amino acid residues 338-393) which is identical among mouse, rat and human (data not shown). Since TRAF6 binds a conserved juxtamembrane region (11), it is unlikely that NADE protein inhibits TRAF6 binding to $p75^{NTR}$. It has been speculated that the polymerization of $p75^{NTR}$ is important for its signal transduction similar to the another members of TNFR family. For example, TNFRI (12), CD40 (13), and Fas (14) are formed the trimer through the binding of each trimer ligands to extracellular domain. However, there was no previous report for $p75^{NTR}$ in same manner (15). It may be possible that the dimer formation of $p75^{NTR}$ occurs through the binding of NADE dimer to its intracellular domain.

To investigate the functional role of NADE protein, NADE and $p75^{NTR}$ were co-transfected in 293T cells. The results showed that the co-transfected 293T cells were detached from the dish and aggregated 48 hours later (Fig. 3a). However, 293T cells transfected with the control plasmid DNAs showed no significant differences (Fig. 3a), implicating that this morphological change is caused by apoptosis. We further examined the TUNEL assay (TdT-mediated dUTP-biotin nick end labeling assay) (16) as well as the DNA fragmentation test on

these cells. On the TUNEL assay, the significant increase of dying cell was detected only in co-transfected cells (Fig. 3b) and the value of the positive cell percentage (38%) was consistent with the transfection efficiency by the calcium-phosphate method. Furthermore, the DNA fragmentation was detected in only the co-transfected 293T cells (Fig. 3c). From these results, we conclude that the co-expression of NADE and $p75^{NTR}$ induced apoptosis in 293T cells.

Although NADE protein is recruited to the cytoplasmic region of $p75^{NTR}$ in a ligand-dependent manner, NGF-dependent cell death was not clearly detected in the co-transfected 293T cells in the presence of NGF (100 ng/ml) (data not shown), suggesting that NADE protein may function in the $p75^{NTR}$-mediated cell death machinery to transduce the downstream signal to apoptosis independent on NGF.

To further investigate the physiological function of NADE protein, we checked the transcription factor kappa B (NF-kB), Caspase-2, and Caspase-3 activities in 293T cells co-transfected with NADE and $p75^{NTR}$. NF-kB is activated by external stimuli, and translocated to the nucleus where it binds to DNA and regulates gene transcription (17). In rat Schwann cells, the binding of NGF to $p75^{NTR}$ induces the activation of NF-kB with independent manner of TrkA (18) leading to the cell survival and TRAF6 may be a component of NGF-mediated NF-kB activation (11). In contrast, expression of NADE protein significantly suppressed the NF-kB activity in a dose dependent manner, but this effect was not markedly co-operative with $p75^{NTR}$ expression (Fig. 3d) as well as NGF-dependent manner (data not shown), implicating that $p75^{NTR}$/NADE-induced apoptosis may not be due to only the suppression of

NF-kB activity but also the regulation of unknown signal molecules since NF-kB suppression by NADE protein alone could not induce apoptosis. It has been reported that suppression of NF-kB activity increases cell death in PC12 cells expressing $p75^{NTR}$ (19, 20). NADE protein may play a key role in the downregulation of NF-kB activity and ultimately lead to apoptosis in neuronal cells expressing $p75^{NTR}$.

In many cases of apoptosis, the elevation of Caspase-3 activity was observed (21, 22, 23, 24). This protease normally exists in cytosol of cells as 32 kDa precursor that is proteolytically activated into a 20 kDa and a 10 kDa hetrodimer when cells are signaled to undergo apoptosis in response to serum withdrawal, activation of Fas, treatment with ionization, and a variety of pharmacological agents (25). Western blot analysis revealed that Caspase-2 and Caspase-3 were significantly processed only in 293T cells co-transfected with NADE and $p75^{NTR}$ (Fig. 3e). Moreover, PARP (poly (ADP-ribose) polymerase) which is a substrate for both Caspase-2 and Caspase-3 were partially cleaved, indicating that these Caspases are involved in apoptosis mediated by $p75^{NTR}$/NADE signal transduction

To investigate whether NES sequences (5) contained in NADE (Fig. 4a) have the capability to export a protein from the nucleus to the cytosol, we performed the transient expression in 293T cells using a series of NADE mutants. The results indicated that NADE proteins with NES sequences localize in the cytoplasmic region (Fig. 4, lower panels of b, upper panels of c and d), but NADE proteins with NES mutations express in the nucleus (Fig. 4, lower panel of c and d). These data support the hypothesis that NADE protein can be exported from the nucleus to the

cytosol and may be post-translationally modified as a prenylated protein to promote and regulate $p75^{NTR}$/NADE physiological interaction.

The signal cascade mediated by $p75^{NTR}$ has been enigmatic for a long time. But the recent growing evidences indicate that, not like other members of TNFR family, $p75^{NTR}$ can bifunctionally mediate signals to induce and inhibit apoptosis (26, 27). Our results strongly supported that NADE is a putative signal transducer for $p75^{NTR}$-mediated apoptosis. Although NADE can mediate apoptosis cooperative with $p75^{NTR}$, it is possible that NADE may be a signal adaptor molecule to interact with another effector molecules in $p75^{NTR}$-mediated signal transduction. More importantly, since NADE has nuclear export signal (NES) as well as ubiquitination sequence, NADE may be tightly controlled by the ubiquitin/proteasome to shuttle another molecule from the nucleus to the cytoplasm, implicating that NADE is a very important protein for turnover of regulator gene such as the cell cycle-related proteins. Further investigation under physiological condition will give us more insight to better understand the mechanisms by which NADE can induce apoptosis together with $p75^{NTR}$ expression.

Methods

Isolation of $p75^{NTR}$-associated cell death executor (NADE) by yeast two-hybrid system.

In order to isolate cDNA encoding $p75^{NTR}$-associated proteins, we used yeast two-hybrid system, originally developed by Fields and Song (28). We used the cytosolic domain of rat $p75^{NTR}$ cDNA corresponding to amino acids 338-396 (representing the cytosolic domain of the protein from the transmenbrane domain to the C-terminus of the protein) as a target. This portion of

$p75^{NTR}$ cDNA was PCR-engineered into the yeast expression plasmid pBTM116 in-frame with sequences encoding the LexA DNA-binding domain (29). This plasmid was then introduced into L40 cells [a, his3, trp1, leu2, ade2, lys2: $(lexAop)^4$-HIS3, URA3: $(lexAop)^8$-lacZ] which contain histidine synthetase (HIS3) and b-galactosidase (lacZ) reporter genes under the control of lexA operators (29). After confirming the expression of LexA-$p75^{NTR}$ (338-396) protein by immunoblotting using an antiserum specific for LexA, a mouse embryo pVP16 cDNA libraries were then introduced into these LexA/$p75^{NTR}$-expressing cells by a high efficiency LiOAc transformation method (30, 31, 32). From a screen of 5 x 10^7 transformants, an initial set of 672 His^+ colonies were identified. These 672 clones were then tested by a β-galactosidase colorimetric assay (33), utilizing the lacZ reporter gene under the control of 8 lexA operators, thus narrowing down the pool of candidate clones to 181, These 181 candidates were then "cured" of their LexA/$p75^{NTR}$-encoding plasmids by growth in tryptophan containing media, and mated with a panel of Mata-type yeast strain NA87-11A [a, leu2, his3, trp1, pho3, pho5] into which we had introduced various control plasmids that produce LexA fusion proteins, including LexA/$p75^{NTR}$, LexA/Ras, Lex/CD40, LexA/Fas, and LexA/lamin. Among the 181 candidate clones, 1 clone specifically reacted with the LexA/$p75^{NTR}$ protein was chosen for further analysis. This mouse cDNA clone No. 59 has insert sizes of 450 bp. Because of its ability to induce cell death with expression of $p75^{NTR}$, we have named this protein, NADE ($p75^{NTR}$-associated cell death executor).

DNA construction.

A full length mouse NADE cDNA was constructed on pBluescript II vector by the ligation of the partial

NADE cDNA (7-524) and 5'-RACE product. PCR cloning techniques were used to replace the stop codon and add the 5' *Xho*I site and 3' *Bam*HI site of a full length NADE cDNA. pcDNA3.1(-)Myc-HisA/NADE was constructed by insertion of a full length NADE cDNA to *Xho*I-*Bam*HI site of pcDNA3.1(-)Myc-HisA (Invitrogen). Human NADE cDNA was amplified using a Jurkat T cell cDNA library and cloned to pcDNA3.1(-)Myc-HisA pcDNA3/rat $p75^{NTR}$ was constructed by insertion of a full length rat $p75^{NTR}$ cDNA to *Eco*RI site of pcDNA3(Invitrogen). pGEX4T-1/rat $p75^{NTR}$ICD was constructed by insertion of amplified rat $p75^{NTR}$ICD(a. a. 338-396) to pGEX4T-1(Pharmacia). Mutant NADE expression plasmids, pcDNA3.1(-)Myc-HisA/muNADE (Cys102Ser) and pcDNA3.1(-)Myc-HisA/muNADE(Cys121Ser), were constructed by PCR-based site-direct mutagenesis methods (29). pELAM-Lu for luciferase reporter assay was constructed by insertion of NF-κB binding site of E-selectin promoter region (-730 - 52) to pGL3-Basic *Sac*I-*Bgl*II site. Expression plasmids of GFP-fused NADE proteins were made following: The cDNA of GFP was cloned into NheI-XhoI-cut pcDNA3.1-mouse NADE as a PCR product amplified with the primers 5"-CTAGCTAGCATCATGGTGAGCAAGGGCGAG-3" and 5"-CCGCTCGAGTCTTGTACAGCTCGTCCAT-3" using pEGFP-N2 (Clontech) as a template. The deletion mutants delta 101-124-GFP and delta 91-124-GFP were constructed by inserting an XhoI-BamHI-cut PCR fragment generated with Expand high fidelity Taq polimerase (Boehringer Mannheim) into XhoI-BamHI-cut pcDNA3.1-GFP using the primers

5"-ATCCTCGAGCGATCATGGCCAATGTCCAC-3" (sense),

5"-ATCGGATCCTCTCAGCTGTAGCTCCCT-3" (antisense) and

5"-ATCGGATCCGATCTCTCTCATCTCCTC-3" (antisense).

The mutagenic primers

5'-AAAGCTTAGGGAGGCACAGCTGAGAAA-3",

5"-TTTCTCAGCTGTGCCTCCCTAAGCTTT-3",
5"-ATCCGGAGAAAGGCTAGGGAGGCACA-3",
and 5"-TGTGCCTCCCTAGCCTTTCTCCGGAT-3")
were used to obtain L97A-GFP and L94, 97A-GFP in which Leu94 and Leu97 are replaced with Ala. In all constructs, mutations were verified by sequencing.

Northern blot analysis. 400 ng of NADE cDNA fragments (nt. 5-510) were labeled by 50 μCi of [a-^{32}P]dCTP and used as a probe. Each 10 μg of total mRNA extracted from mouse various tissues were transferred on membranes and they were hybridized with a NADE probe for 2 hours at 68 °C using a express hybrid buffer (Clontech) and washed with 2 x SSC, 0.05 % SDS for 5 times, and 0.1 x SSC, 0.1 % SDS for 1 time.

Antibodies. The polyclonal anti-NADE antibody was prepared by immunization of GST-mouse NADE fusion protein into the rabbit. The NADE specific antibody was affinity purified by antigen coupled Sepharose 4B. The polyclonal anti-rat p75^{NTR} was kindly gifted from Dr. M. V. Chao. The monoclonal anti-Myc antibody (9E10) was purchased from BIOMOL. The polyclonal anti-Caspase-3 antibody (H-277) was purchased from Santa Cruz Biotechnology. The polyclonal Caspase-2 antibody was kindly gifted from Dr. Lloyd A. Greene. HRP conjugated anti-rabbit IgG was purchased from Bio-Rad.

Immunoprecipitation and immunoblotting. In Fig. 1e, 150 μg/ml of ALLN (N-Acetyl-Leu-Leu-Norleucinal) treated SK-N-MC cells (1 x 10^7) were lysed in 0.5 ml of RIPA buffer. The supernatant of centrifuge (100,000 x g) was mixed with 1 μg of polyclonal anti-NADE antibody coupled Sepharose 4B, and incubated for 4 hours at 4 °C. After washing, the gels were boiled by 30 μl of SDS-PAGE sampling buffer and subjected to

12.5 % of SDS-PAGE. Immunoblotting was performed by polyclonal anti-NADE antibody (2 μg/ml). In Fig. 1f, 10 μg of cell lysate extracted from each transfected 293T cells were used for the detection of NADE by immunoblotting.

Transfection and protein expression in 293T cell. In Fig. 1f, 293T cells (2 x 10^6) were transfected by 10 μg of pcDNA3.1(-)Myc-HisA/NADE, pcDNA3.1(-)Myc-HisA/muNADE (Cys102Ser), or pcDNA3.1(-)Myc-HisA/muNADE(Cys121Ser) by calcium-phosphate method. In Fig. 2 b, 3 a, b, c, e, 293T cells (2 x 10^6) were transfected by 20 μg of pcDNA3.1(-) Myc-HisA, μ10 g of pcDNA3/rat $p75^{NTR}$ and 10 μg of pcDNA3.1(-) Myc-HisA, 10 μg of pcDNA3.1(-)Myc-HisA NADE and 10 g of pcDNA3.1(-) Myc-HisA, or 10 μg of pcDNA3.1(-)Myc-HisA/NADE and 10 μg of pcDNA3 / rat $p75^{NTR}$. In Fig. 2 c, 293T cells (2 x 10^6) were transfected by 10 μg of pcDNA3.1(-)Myc-HisA/NADE and 10 μg of pcDNA3/rat $p75^{NTR}$ in serum minus DMEM medium.

***In vitro* binding assay.** 5 μl of L-[^{35}S] methionine labeled, and *in vitro*- translated NADE protein was mixed with 5 μl of GST-rat $p75^{NTR}$ICD fusion protein or GST-coupled GSH-Sepharose 4B (Pharmacia) in 100 μl of NETN buffer (20 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.2 % NP-40) for 18 hours at 4 °C. After washing, gels were boiled by 30 μl of SDS-PAGE sampling buffer and subjected to 13.5 % SDS-PAGE. The fluolography was performed for 16 hours at -70 °C.

***In vivo* binding assay.** In Fig. 2b, transfected 293T cells by were lysed in 1 ml of NETN buffer and centrifuged (100,000 μg). The supernatants were immunoprecipitated by 2 μg of anti-Myc antibody coupled Protein G Sepharose 4B (Pharmacia) for 2 hours at 4 °C. Following the 5 times washing, gels were

subjected to 7.5 % SDS-PAGE, and Western blot analysis by rabbit polyclonal anti-$p75^{NTR}$ antibody.

Interaction of NADE with $p75^{NTR}$ dependent on NGF ligation. After co-transfection, cells were incubated in DMEM medium containing various NGF. After 12 hours later, the interaction activity between NADE and $p75^{NTR}$ were checked by *in vivo* binding assay.

TUNEL assay. MEBSTAIN Apoptosis kit direct (MIC) was used for TUNEL assay and the assay was done according to the company instruction. The stained cells were analyzed by FACSCalibur flow cytometer (Becton Dickinson).

DNA fragmentation assay. Transfected 293T cells were lysed in 350 μl of 10 mM EDTA and 0.5 % SDS for 10 minutes at room temperature. After adding 100 μl of 5 M NaCl, the aliquot was incubated for 18 hours at 4 °C and centrifuged (12,000 x g). The supernatants were treated by 1 mg/ml of RNase A and 50 ng/ml of Proteinase K for 2 hours at 42 °C. After the phenol-chloroform extraction, the DNAs were precipitated by 70 % ethanol, and dissolved in 30 μl of H_2O. 5 μl of samples were subjected to the 1.5 % agarose gel electrophoresis.

Measurement of NF-B activity. Dual-Luciferase Reporter Assay System (Promega) was used for measurement of NF-κB activity. 293T cells (4×10^5) were transfected with 1.5 μg of pELAM-luc reporter plasmid, 0.1 μg of pRL-TK, 0.7 μg of pcDNA3 rat $p75^{NTR}$, 0.3 μg or 2.8 μg of pcDNA3.1(-) Myc-HisA/NADE and enough pcDNA3.1(-) Myc-His a control plasmid to give 5.1 μg of total DNA. Luciferase activities were determined 24 hours after transfection and normalized on the basis of pRL-TK expression levels. The

luciferase activities were measured by Turner Designs Luminometer Model TD20/20 (Promega).

Confocal laser microscopy

Transient transfections were carried out using the calcium phosphate precipitation method. 293T cells ($3X10^5$) on a cover glass were transiently transfected with 3.0 ug of DNA. After 12-24 hours, cells were fixed with 4 % paraformaldehyde and stained with TO-PRO-3 Iodide (Molecular Probes, Inc.) to visualize the nucleus. The subcellular distribution of GFP fusion proteins was examined using confocal laser microscopy (Carl Zeiss LSM510).